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Role of Viscoelasticity in Bacterial Killing by Antimicrobials in Differently Grown *P. aeruginosa* Biofilms

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ABSTRACT

Pseudomonas aeruginosa colonizes the sputum of most adult cystic fibrosis patients, forming hard to eradicate biofilms, in which bacteria are protected in their self-produced EPS-matrix. EPS provides biofilms with viscoelastic properties, causing time-dependent relaxation after stress-induced deformation, according to multiple characteristic time-constants. These time-constants reflect different biofilm (matrix) components. Since viscoelasticity of biofilms has been related with antimicrobial penetration, but not yet with bacterial killing, this study aims to relate killing of *P. aeruginosa* in its biofilm-mode of growth by three antimicrobials with biofilm viscoelasticity. *P. aeruginosa* biofilms were grown for 18 h in a constant depth film fermenter, either with mucin-containing artificial sputum medium (ASM^+), artificial sputum medium without mucin (ASM^-), or Luria-Bertani broth (LB). This yielded 100 µm thick biofilms, that differed in their amounts of matrix eDNA and polysaccharides. Low-load-compression-testing followed by three-element Maxwell analyses, showed that the fastest relaxation component, associated with unbound water, was most important in LB-grown biofilms. Slower components due to water with dissolved polysaccharides, insoluble polysaccharides and eDNA, were most important in relaxation of ASM^+-grown biofilms. ASM^--grown biofilms showed intermediate stress relaxation. *P. aeruginosa* in LB-grown biofilms were killed most by exposure to tobramycin, colistin or an antimicrobial peptide, while ASM^+ provided the most protective matrix with less water and most insoluble polysaccharides and eDNA. Concluding, stress relaxation of *P. aeruginosa* biofilms grown in different media revealed differences in matrix composition that, within the constraints of the antimicrobials and growth media applied, correlated with the matrix protection offered against different antimicrobials.

KEYWORDS biofilm recalcitrance, biofilm matrix, extracellular polymeric substances (EPS), antimicrobial penetration, cystic fibrosis, artificial sputum medium

INTRODUCTION

Gram-negative *Pseudomonas aeruginosa* biofilms play an important role in chronic wound infections, otitis media, biomaterial-associated infections and cystic fibrosis (CF) pneumonia (1). CF is characterized by the formation of thick mucus layers in the lungs, which makes it a suitable environment for *P. aeruginosa* to form biofilms (2). Approximately 80% of all adult CF patients are chronically infected by mucoid *P. aeruginosa*, which results in chronic illness and potentially death (3). Biofilm infections, including CF, are difficult to treat because the infecting bacteria surround themselves in a self-produced matrix of extracellular polymeric substances (EPS) (4). This can result in up to 1000 times larger recalcitrance to antimicrobials than planktonic bacteria possess (5). Multiple mechanisms have been described for this recalcitrance of bacteria in a biofilm-mode of growth, such as reduced metabolic activity, presence of persister cells and hampered penetration of antimicrobials into biofilms (3). The EPS matrix in *P. aeruginosa* biofilms mainly consists of water, proteins, lipids, eDNA and polysaccharides (6). The hallmark of CF infections caused by *P. aeruginosa* is the overproduction of polysaccharides, which negatively impacts survival of CF patients (7) as it facilitates strong bacterial binding and therewith hampering clearance from the lungs as well as providing protection against the host immune system and antimicrobials.

EPS provides biofilms with viscoelastic properties. Elasticity relates to an immediate return of a material to its original shape after stress application, while viscoelasticity is the time-dependent, partial resumption of the original shape of a material after being deformed. The time-dependent resumption of the biofilm shape after stress application can be subjected to a Maxwell

analysis (8–10) to identify different stress relaxation processes occurring in a biofilm. Empirically, stress relaxation in biofilms has been divided in a fast relaxation (stress relaxation time range 0 - 5 s) due to fast flow of water with its low viscosity, a slow component (> 100 s) related to re-positioning of bacterial cells and an intermediate component (5 - 100 s) caused by flow of more viscous EPS. Chlorohexidine penetration and bacterial killing in oral biofilms related with biofilm viscoelasticity, decreasing with increasing prevalence of the fastest, water-due component and increasing with decreasing prevalence of the slowest component associated with bacterial re-arrangement (8). Accordingly, viscoelasticity of a biofilm has been called a virulence factor (11). More detailed principal component analysis attributed stress relaxation time ranges to three principal components due to water and soluble polysaccharides (0.01 – 3 s), EPS components, like insoluble polysaccharides (3 – 70 s), comprising a principal component exclusively due to eDNA (10 – 25 s) (9). Collectively, these relatively fast components related inversely with slow stress relaxation possessing time range constants >70 s, while being due to bacterial cell re-arrangement (9).

However, although the viscoelastic properties of biofilms have been related to the combined effects of antimicrobial penetration and killing that jointly define “recalcitrance”, no direct relation between the viscoelasticity of a biofilm and antimicrobial killing has been established. Therefore, the aim of study is to relate the viscoelasticity of *P. aeruginosa* biofilm with the killing of biofilm inhabitants by tobramycin, colistin or an antimicrobial peptide at different concentrations. To this end, *P. aeruginosa* biofilms were grown in a constant depth film fermenter (CDFF) in mucin containing artificial sputum medium (ASM^{+}), bearing similarity to the lung environment (12), artificial sputum medium without mucin (AMS^{-}) or Luria-Bertani broth (LB), a high-nutrient, standard laboratory medium. Viscoelasticity of the biofilm will be

determined from the stress relaxation of deformed biofilm and subsequent Maxwell analyses of the relaxation time-constants.

RESULTS

Growth rate and antimicrobial susceptibility of planktonic *P. aeruginosa* in different media. No differences were observed in the growth rate of planktonic, mucoid *P. aeruginosa* ATCC 39324, a clinical CF isolate, when bacteria were grown in ASM^+, ASM^-, or LB (Fig. 1a). The minimal bactericidal concentrations (MBC) against planktonic *P. aeruginosa* ATCC 39324 grown in different media are shown in the Table inset to Fig. 1 (Fig. 1b). Tobramycin and colistin yielded the same MBC regardless of the growth medium used, but for the antimicrobial peptide AA-230, the MBC of *P. aeruginosa* grown in ASM^+ and ASM^- was 4 times higher than of bacteria grown in LB.

Characteristics and matrix composition of differently grown *P. aeruginosa* biofilms. Biofilms of *P. aeruginosa* ATCC 39324 were grown in a constant depth film fermenter (CDFF) with ASM^+, ASM^- and LB for 18 h, employing wells with a depth of 100 µm (13). Biofilms were imaged using optical coherence tomography (OCT) and using confocal laser scanning microscopy (CLSM) after staining (Figs. 2a and 2b, respectively). 2D cross-sectional OCT images (Fig. 2a) confirmed that on average, all biofilms grown were 100 µm thick (Fig. 3a), irrespective of the growth medium applied. Standard deviations over the thickness of biofilms grown in ASM^+ medium with a surplus of mucin (22%; over three different CDFF runs, taking 10 biofilms out of each run) were on average two-fold larger than of biofilms grown in absence of a surplus of mucin (11% in both ASM^- and LB medium). In CLSM images, biofilms grown with ASM^+ showed a heterogeneous distribution of microcolonies surrounded by microchannels (Fig. 2b). Biofilms grown with LB had a highly homogeneous structure, without microcolonies

and less obvious microchannels. Biofilms grown with ASM$^-$ displayed an intermediate structure compared to the biofilms in ASM+ and LB media. COMSTAT analysis demonstrated no significant differences in biovolume of the biofilms (Fig. 3b). Metabolic activity of biofilms grown with ASM$^+$, ASM$^-$ and LB also showed no significant differences (Fig. 3c). Concentrations of eDNA (Fig. 3d) were similar in ASM$^+$ and ASM$^-$ biofilms and higher than in the LB biofilms. Polysaccharides concentrations (Fig. 3e) were similar in ASM$^-$ and LB biofilm, while highest in the ASM$^+$ biofilm. No differences were found in protein concentration (Fig. 3f) and water content (Fig. 3g). Significant differences in *P. aeruginosa* biofilm characteristics are summarized in Table 1.

Viscoelastic properties of differently grown *P. aeruginosa* biofilms. Biofilms were compressed within 1 s to 80% of their initial thickness, equivalent to a strain (ε) of 0.2. Normalized stress on the biofilms required to maintain the same deformation decreased with time (Fig. 4a), showing relatively slow stress relaxation for ASM$^+$ grown biofilms, while LB grown biofilms relaxed fastest. All biofilms showed near full stress relaxation towards 100 s. Stress relaxation as a function of time was fitted to a three element Maxwell model. Inclusion of more Maxwell elements did not yield a better quality of the fit (Fig. 4b). LB grown biofilms showed a significantly higher relative importance of the fastest time constant range (< 0.75 s), than ASM$^+$ and ASM$^-$ grown biofilms, with ASM$^+$ grown biofilms showing the lowest relative importance of the fastest relaxation time range (Fig. 4c). Relative importance of the other relaxation time constants ranging up to 25 s, was highest for ASM$^+$ and lowest for LB grown biofilms (see also Table 1).

Antimicrobial killing in differently grown *P. aeruginosa* biofilms. Biofilms were exposed for 24 h to PBS or PBS containing tobramycin, colistin or the antimicrobial peptide AA-230 at concentrations well above their MBC towards planktonic *P. aeruginosa* ATCC 39324 (see

Fig. 1B). Tobramycin concentrations applied were 1000, 2500 and 5000 µg/ml, equivalent to 62x, 156x and 313x MBC, respectively. Colistin was applied at concentrations of 1000 and 2500 µg/ml, equivalent to 8x and 20x MBC, respectively. For tobramycin and colistin, MBC-fold concentrations were independent of growth medium (see also Fig. 1b), but for the antimicrobial peptide AA-230 the concentrations applied (5000 and 10,000 µg/ml) yielded different MBC-fold concentrations for bacteria grown in ASM^+ or ASM^- media (39x and 78x MBC, respectively) than for bacteria grown in LB medium (156x and 313x MBC, respectively). After antimicrobial exposure, biofilms were dispersed and the number of CFUs in the biofilms counted, taking PBS as a control. Control biofilms contained on average 1.8×10^9 CFU/cm^2, regardless of the growth medium applied (Fig. 5). All antimicrobial exposures resulted in a significant decrease in CFU/cm^2, as compared to biofilms after PBS exposure, with a clear dose response. In general, ASM^+ and ASM^- grown biofilms showed significantly higher numbers of CFUs, i.e. lower killing by antimicrobials than LB grown biofilms. No significant differences were observed between ASM^+ and ASM^- grown biofilms.

DISCUSSION

This study demonstrates that *P. aeruginosa* ATCC 39324 biofilms grown to a thickness of 100 µm in a CDFF possess different matrix compositions when grown in different growth media (Fig. 3) and allow different degrees of killing of its bacterial inhabitants by antimicrobials (Fig. 5). Maxwell analyses showed that the fastest relaxation component, associated with unbound water, was most important in LB grown biofilms (Fig. 4c), but in absence of obvious microchannels (Fig. 2b). Slower stress relaxation components due to water with dissolved polysaccharides, insoluble polysaccharides and eDNA were most important in relaxation of ASM^+ grown biofilms. ASM^- grown biofilms showed intermediate stress relaxation. *P.*

aeruginosa in LB grown biofilms were killed most by exposure to tobramycin, colistin or an antimicrobial peptide, possibly due to the transport options provided by water. Biofilm growth in ASM^+ provided the most protective matrix with less unbound water and most insoluble polysaccharides and eDNA, that maximally hampered penetration and killing. Interestingly, this statement coincides with the observation of microchannels in ASM^+ grown biofilm (Fig. 2b). Since microchannels by definition have a transport function (14), this suggests that matrix composition may be more important than the possession of clear channel-like structures for the transport of antimicrobials in a biofilm. Concluding, stress relaxation analysis (Fig. 4) of *P. aeruginosa* biofilms grown in different media revealed differences in matrix composition (Fig. 3) that, within the constraints of the antimicrobials and growth media applied, correlated with the matrix protection offered against different antimicrobials (Fig. 5). Without the use of a CDFF, it would have been impossible to carry out this study because use of different growth media would have yielded biofilms with different thickness (15–18).

Two artificial sputum media were used, mimicking the environment of the lung of CF patients and a nutrient-rich, laboratory medium (LB). In artificial sputum media, biofilms possessed more matrix eDNA and polysaccharides than biofilms grown in LB. The possession of more eDNA and polysaccharides yielded different stress relaxation behavior of the biofilms, with a higher importance of time relaxation ranges between 3 to 10 s and 10 to 25 s, respectively. This is fully in line with previous analyses of stress relaxation time ranges of biofilms from a wide variety of different strains and species with known matrix amounts of eDNA and polysaccharides (9). Both eDNA and polysaccharides act as a glue in biofilms and growth in artificial sputum medium accordingly gave more compact biofilms with condensed microcolonies in comparison with LB grown biofilms (Fig. 2b), in line with literature (15, 19). The arrangement of bacteria in more compact, condensed microcolonies limits their possibility to re-arrange during stress

189 relaxation, which explains their slower relaxation (Fig. 4a). Although growth media were selected
190 that mimic the environment of the lungs, it is virtually impossible to select a substratum material
191 to grow the biofilms upon that also mimics the *in vivo* situation. However, considering the use of
192 CDFF-grown biofilms, compressed by the scraper-action and the limited calling distance of
193 quorum-sensing molecules in a biofilm, it may be expected that over the 100 µm thickness of the
194 Pseudomonas biofilms studied, influences of the substratum may have averaged out (20).

195 LB grown biofilms demonstrated a stronger influence of water (relaxation times < 0.75 s)
196 than biofilms grown in artificial sputum media (see also Table 1), although dry weight
197 measurement of the percentage water in the biofilms were too insensitive to reflect differences
198 with biofilms grown in other media (see Fig. 3a). Note that previously, relaxation time constants
199 up to 3 s were taken together (9), while we here separate this relaxation time constant range into
200 two relaxation time ranges, attributing the time constant range between 0.75 and 3 s to more
201 viscous water with dissolved polysaccharides. Through this distinction, the role of undissolved
202 polysaccharides as a glue becomes reflected in stress relaxation analysis of biofilms.

203 Tobramycin, colistin and AA-230 are all hydrophilic antimicrobials and have a similar
204 positive charge at physiological pH (21–23). The molecular weight of tobramycin is 468 g/mol,
205 of colistin is 1156 g/mol and of AA-230 2578 g/mol. This makes it interesting to compare
206 bacterial killing by these antimicrobials at equivalent molar concentrations. At an equivalent
207 molar concentration between 1.9 and 2.1 µM, corresponding with tobramycin, colistin and AA-
208 230 concentrations of 1000, 2500 and 5000 µg/ml respectively, colistin showed higher killing
209 than tobramycin and AA-230, which is unexpected (24) because colistin with its higher molecular
210 weight will diffuse more slowly into a biofilm at a similar molar concentrations. Moreover,
211 expressing antimicrobial concentrations in MBC-fold equivalents, a molar concentration of 2.1
212 µM colistin corresponds with an MBC-fold concentration of only 20x MBC, far lower than

MBC-fold concentrations of tobramycin or AA-230 at a similar molar concentration. Likely, these differences in killing efficacy are due to their different modes of killing: tobramycin works on the inhibition of protein synthesis, and colistin on the destruction of the bacterial cell membrane (22, 25). The antimicrobial peptide AA-230 with the highest molecular weight, has a similar mode of action and accordingly showed less killing than colistin due to hampered penetration. Penetration of all three cationic antimicrobials will likely be hampered by the high concentrations of eDNA and polysaccharides in the artificial sputum grown biofilms due to electrostatic double-layer attraction with negatively charged bacterial cell surface components (23, 26–29). Moreover, eDNA in the biofilms induces production of spermidine and amino-arabinose on the outer membrane, thereby reducing the permeability to aminoglycosides such as tobramycin (27) and leading to a decreased killing efficacy in biofilms grown in the artificial sputum media. In addition, the presence of mucin also can give rise to a higher tolerance for tobramycin (19). However, this effect is probably of minor importance, as differences in killing of *P. aeruginosa* biofilms grown in artificial sputum medium with or without mucin are small (see also Fig. 5).

In conclusion, 1) viscoelastic properties of *P. aeruginosa* biofilms grown in a CDFF and in different media differ due to possession of different amounts of water, (in)soluble polysaccharide and eDNA concentrations and 2) within the constraints of the antimicrobials and growth media applied, these properties relate with antimicrobial bacterial killing in the biofilm. More unbound water and less EPS, i.e. polysaccharides and eDNA, as in LB-grown biofilms facilitated higher killing than a less aqueous matrix with more EPS, as in ASM^{+}-grown biofilms.

MATERIALS AND METHODS

Bacterial cultures and medium. *P. aeruginosa* ATCC 39324, a clinical CF isolate (mucoid phenotype) was cultured on a blood agar plate and a single colony was used to inoculate 10 ml of tryptone soya broth (TSB, OXOID, Basingstoke, England) for aerobic incubation at 37°C. After 24 h, this pre-culture was added to 200 ml of TSB and incubated aerobically at 37°C for 16 h under rotary-shaking at 150 revolutions per min (RPM), after which bacteria were harvested by centrifugation (5,000 X *g*, 5 min, 10°C). Bacterial pellets were washed two times with 10 ml sterile phosphate buffered saline (PBS, 10 mM potassium phosphate, 150 mM NaCl, pH 7.0) and bacteria were resuspended in 10 ml sterile PBS and bacterial concentrations determined using a Bürker-Türk counting chamber.

Planktonic growth rate. In order to investigate whether *P. aeruginosa* growth rates were similar in the different media, bacteria were suspended to 10^4 CFU/ml in 40 ml of artificial sputum medium (12) (ASM^+: per liter: 4 g DNA, 5 g mucin, 5 ml egg yolk emulsion, 4.75 g casamino acids, 0.25 g L-tryptophan, 5 g NaCl, 2.2 g KCl, pH 7.0), artificial sputum medium without mucin (ASM^-), and LB, and incubated at 37°C under rotary-shaking at 150 RPM. At time 0 and after 3, 6, and 24 h, 200 µl aliquots were taken and 10-fold serially diluted in sterile PBS. Two 10 µl droplets of each dilution were spotted on a tryptone soya agar plate and incubated 24 h at 37°C, after which the numbers of colony forming units were counted and expressed as CFU/ml.

Minimal bactericidal concentration (MBC). Two-fold serial dilutions from 512 to 1 µg/ml of the antimicrobials were made in a 96 wells plate in ASM^+, ASM^- and LB, each with a total volume of 100 µl. For control, wells filled with growth medium in absence of antimicrobials were used. Bacteria were diluted to a concentration of 2×10^6 bacteria/ml in either ASM^+, ASM^- or LB and 100 µl of bacterial suspension was added to each well to yield a total volume in the well of 200 µl. The plates were incubated statically at 37°C, and after 24 h 30 µl aliquots were

spotted on tryptone soya agar plates, after which the plates were incubated at 37°C for 24 h. The lowest antimicrobial concentration not showing visible bacterial colonies was taken as the MBC.

Biofilm growth. Biofilms were grown in a sterile constant depth film fermenter (CDFF) (13, 30) at 37°C on stainless steel disks. The sterile disks were placed in each of the 5 wells of a pan, placing 15 pans in the turntable of the CDFF. The thickness of the biofilms in the pans was controlled by setting the well depth such to leave 100 µm above the disks for the biofilm to grow. An amount of 200 ml bacterial suspension in TSB containing 5×10^7 bacteria/ml was introduced in the CDFF during1 h, while the turntable was rotating at 3 RPM. Rotation was stopped for 30 min to allow bacterial adhesion before the growth medium (ASM^+, ASM^- or LB) was introduced and rotation continued. The biofilm was grown for 18 h and medium flow was continuous at a flow rate of 16 ml/h. After 18 h, disks with adhering biofilms were aseptically taken out of the pans for further experiments.

Miscellaneous properties of differently grown biofilms. To determine the average thickness of biofilms, OCT (Ganymede-II, Thorlabs, Lubeck, Germany) was used. Biofilms were submerged in PBS, and a series of 2D, cross-sectional images of the biofilms were generated (1500 x 372 pixels). The average thickness of the biofilm was derived from the 2D cross-sectional images using Otsu-thresholding (31).

Biofilms were stained using SYTO9 (*bac*Light™, Invitrogen, Breda, The Netherlands) live stain for 30 min in the dark to reveal biofilm structure. After staining, biofilms were immersed in PBS and images were taken with a confocal laser scanning microscope (CLSM, Leica TCS-SP2, Leica microsystems GmbH, Heidelberg, Germany) with a 40× water objective lens. Images were analyzed using Fiji (32), Imaris (Bitplane, Belfast, UK) and COMSTAT 2.1 (33, 34).

283 In order to investigate whether *P. aeruginosa* biofilms grown in the different media had
284 similar metabolic activity, biofilms were exposed to 200 µl (3-(4,5-Dimethylthiazol-2-yl)-2,5-
285 Diphenyltetrazolium Bromide (MTT, 0.75 mg/ml) dissolved in sterile PBS at 37°C for 2.5 h. In
286 metabolic active bacteria, MTT is intracellularly reduced to formazan. Following the incubation,
287 biofilms were washed using PBS, and 1 ml of isopropanol was added to dissolve the formazan
288 crystals inside the bacterial cells, and the optical density of the solution was measured at 575 nm
289 using a FLUOstar optima plate reader (BMG Labtech GmbH, Offenburg, Germany).

290 **Matrix composition.** For eDNA concentration, 1 ml eDNA extraction buffer (10 mM
291 EDTA, 0.9% NaCl) was added to individual biofilms taken over three CDFF runs and 5 biofilms
292 per CDFF run, vortexed and resuspended until the biofilm was fully detached from the
293 substratum disk. Dispersed biofilms were centrifuged (5000 X *g*, 5 min, 10°C) to remove intact
294 bacterial cells along with intracellular DNA. eDNA isolation was done using the phenol
295 chloroform method (9). RNase was added to the eDNA after isolation and incubated for 30 min at
296 37°C, after which the concentration of eDNA was measured using the ratio of absorbance at 260
297 nm and 280 nm with the nanodrop-method.

298 For both polysaccharides and protein determination, five biofilms from different pans
299 within one CDFF run were pooled and resuspended in 500 µl sterile PBS and vortexed for 1 min
300 to detach the biofilms from the substratum disks. This was done in triplicate, with separated
301 CDFF runs. Resuspended biofilms were centrifuged (5,000 X *g*, 5 min, 10 °C) to remove
302 bacterial cells, after which 400 µl of supernatant was collected. Supernatant was immediately
303 placed on ice and used for both polysaccharides and protein determination.

304 Protein concentration was measured with the Pierce BCA Protein Assay Kit (Thermo
305 Scientific, Waltham, USA), using the microplate procedure. Briefly, 25 µl sample from the
306 supernatant or bovine serum albumin (2000 to 25 µg/ml bovine albumin used for the calibration

curve) was added to 200 µl working reagent, and mixed for 30 s. Plates were incubated for 30 min at 37°C, after which plates were cooled down to room temperature, and absorbance at 560 nm was measured using a FLUOstar optima plate reader.

Polysaccharide determination was performed using the colorimetric assay for glucose-based carbohydrates (35). For the glucose based carbohydrates method, 40 µl of supernatant or a glucose solution (4096 to 1 µg/ml used for the calibration curve) was added to a 96-well plate and placed in the refrigerator for 15 min. 100 µl of anthrone solution (2 mg/ml in H_2SO_4) was added to the wells, mixed, and incubated for 3 min at 92°C, after which plates were placed in a water bath at room temperature for 15 min. Absorbance at 590 nm was measured and compared against glucose calibration curve with a FLUOstar optima plate reader

To determine the water percentage in the biofilms, the wet weight of the disk and biofilm was measured using an analytical balance (Mettler Toledo model XP105DR, Columbus, USA). Prior to weighing, the bottom and the sides of the disks were carefully dried with a tissue, after which their weights were measured. Then, the disks with biofilms were dried at 60°C in a vacuum oven for at least 24 h, and weighted again. Afterwards the dried biofilm was removed from the disk and the disk alone was weighted. With this data, the dry weight and water percentage of the biofilms was calculated.

Viscoelastic properties of *P. aeruginosa* biofilms. Viscoelastic properties of the biofilms were determined by using the low-load-compression-tester (LLCT) (36). To this end, a small part of a substratum disks was cleaned to enable determination of the substratum surface using LLCT plunger (2.5 mm diameter). After determining the position of the substratum surface, the position of the biofilm surface was determined by compressing the biofilm with a small "touch level" of 0.01 g. Next, the biofilm was compressed to 80% of its original thickness (strain 0.2) within 1 s

and held constant for 100 s, while measuring stress relaxation. Stress relaxation ($\sigma(t)$) was measured as a function of time t during 100 s and normalized with respect to strain according to

$$E(t)=\frac{\sigma(t)}{\varepsilon} \quad (1)$$

Normalized stress relaxation as a function of time $E(t)$ was fitted to a three element, generalized Maxwell model, using the solver tool in Microsoft Excel 2010. according to

$$E(t)=\frac{\sigma(t)}{\varepsilon}=E_1 e^{-t/\tau_1}+E_2 e^{-t/\tau_2}+E_3 e^{-t/\tau_3} \quad (2)$$

where $\tau_i = \eta_i/E_i$ is the relaxation time constant, E_i is the spring constant and η_i is the viscosity term for each Maxwell element i. In order to indicate the relative importance of each relaxation process RI_i, defined as

$$RI_i=E_i/((E_1+E_2+E_3)\times 100\% \quad (3)$$

was calculated for each element and elements were placed in six relaxation time ranges.

Antimicrobial exposure of *P. aeruginosa* biofilms and bacterial killing. Biofilms were exposed to different concentrations of tobramycin (5000, 2500, 1000 µg/ml) or colistin sodium methanesulfonate ("colistin", 2500, 1000 µg/ml), both purchased from Sigma-Aldrich Chemie

GmbH (Steinheim, Germany), and the antimicrobial peptide AA-230 (10000, 5000 µg/ml), supplied by Adenium Biotech (Copenhagen, Denmark) and synthesized by PolyPeptide (Malmö, Sweden). Tobramycin and colistin are both used against *P. aeruginosa* infections in CF patients. All antimicrobials were dissolved in sterile PBS. A 20 µl drop of sterile PBS or antimicrobial solution was pipetted on the biofilm. The plate was sealed with parafilm to prevent evaporation and incubated 24 h at 37°C. Concentrations were chosen well above the MBC of each antimicrobial against planktonically-grown *P. aeruginosa*. After antimicrobial exposure, biofilms were washed with sterile PBS, 1 ml sterile PBS added and vortexed for 1 min, to disrupt the biofilm and detach the bacteria from the substratum disk. Finally, dispersions were sonicated in a sonication bath for 5 min to disrupt bacterial aggregates, which did not cause bacterial death (data not shown). Samples were 10-fold serially diluted and two 10 µl drops of each dilution were spotted on tryptone soya agar and incubated at 37°C. After 24 h, colonies were counted and the number of CFU per cm^2 substratum surface calculated.

Statistical analysis. Statistical analysis was performed with Graphpad Prism version 5.00 for Windows (GraphPad Software, La Jolla California USA). Differences in biofilm thickness, viscoelastic properties, biofilm recalcitrance to antimicrobials and EPS components were evaluated after normality testing. Analysis of variance (ANOVA) was performed to test significance between groups, either with a Dunn's Post-hoc test or a Tukey post-hoc test, depending on normal or non-normal distribution. Data was accepted significant if $p < 0.05$. All data reported represent means with standard deviation, unless stated otherwise.

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AUTHOR CONTRIBUTIONS

RTR and WW collected and analyzed the data presented. RTR, HCVDM, HJB and PKS prepared the outline of the manuscript and wrote the text. The text was critically reviewed by WW and EDdJ.

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HJB is also director of a consulting company, SASA BV (GN Schutterlaan 4, 9797 PC Thesinge, The Netherlands). The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of their respective employers.

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492 **TABLE 1** Summary of the statistically significant differences in *P. aeruginosa* ATCC 39324
493 biofilm characteristics grown in ASM^+, ASM^- or LB media and their killing by antimicrobials
494 (taking tobramycin, colistin and the antimicrobial peptide AA-230 together). = means no
495 significant difference, > means significant difference at $p < 0.05$.

Biofilm characteristic	**No difference/ difference**
Matrix eDNA	$ASM^+ = ASM^- > LB$
Matrix polysaccharides	$ASM^+ > ASM^- = LB$
Stress relaxation time < 0.75 s	$LB > ASM^- > ASM^+$
0.75 s < Stress relaxation time < 3 s	$ASM^+ > ASM^- > LB$
3 s < Stress relaxation time < 10 s	$ASM^+ > LB$; $ASM^+ =$ ASM-; $LB = ASM^-$
10 s < Stress relaxation time < 25 s	$ASM^+ > LB$; $ASM^+ = ASM^-$; $LB = ASM^-$
Antimicrobial killing	$LB > ASM^- \geq ASM^+$

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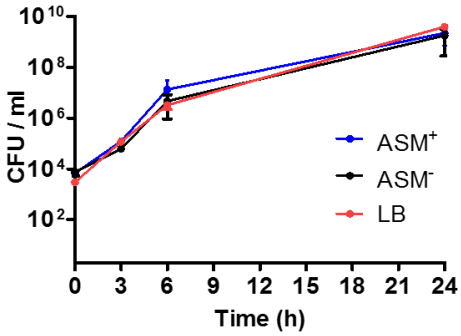
a



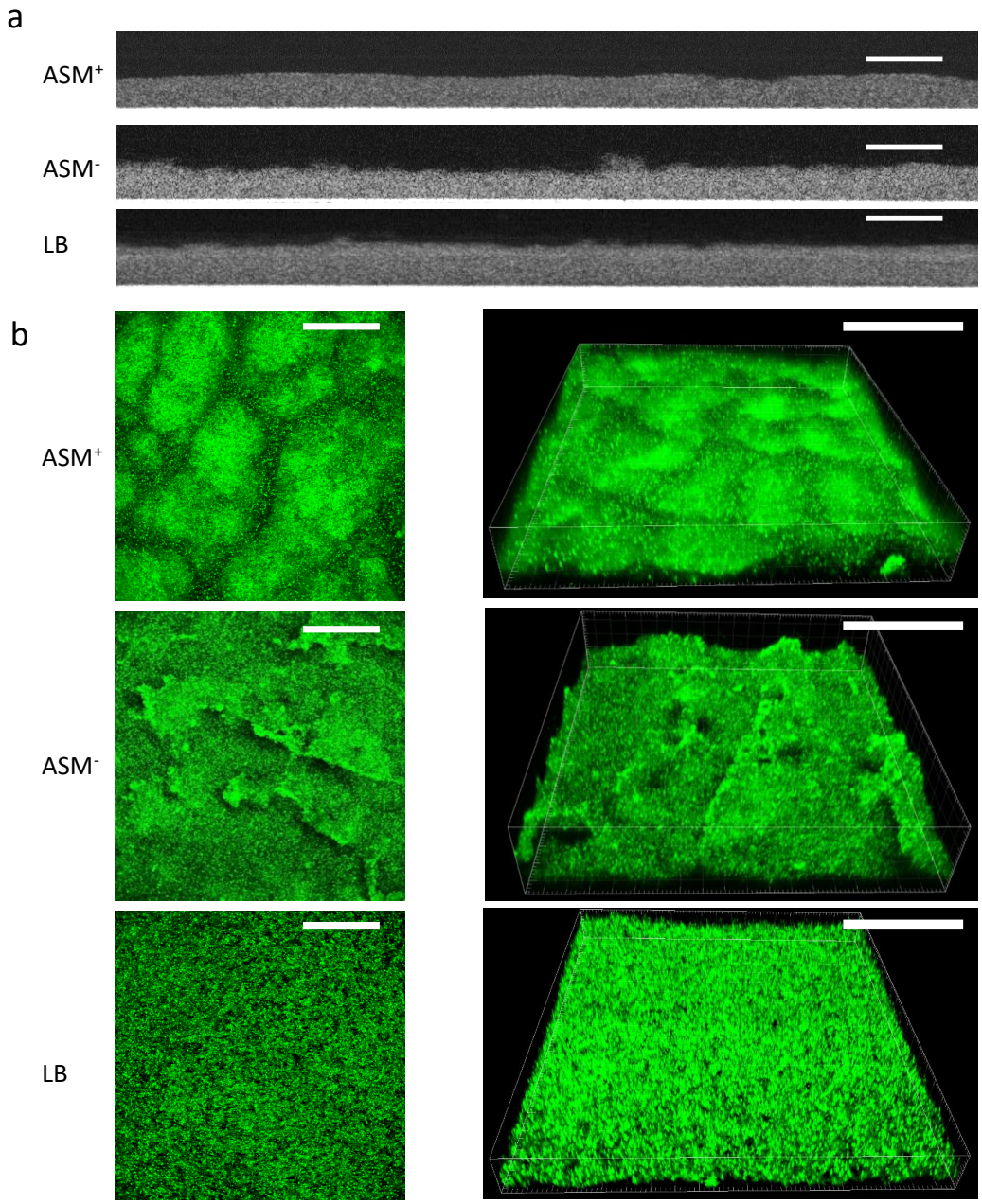
b

Antimicrobial agent	MBC in ASM$^+$ (µg/ml)	MBC in ASM$^-$(µg/ml)	MBC in LB (µg/ml)
Tobramycin	16	16	16
Colistin	128	128	128
AA-230	128	128	32

FIG 1 Planktonic growth curve and minimal bactericidal concentration (MBC) of *P. aeruginosa* ATCC 39324 in ASM$^+$, ASM$^-$ and LB medium.

(a) The number of CFU/ml in planktonic cultures as a function of time in different growth media. Growth curves were done in duplicate, error bars denoting the difference between the two experiments.

(b) MBC of planktonic *P. aeruginosa* upon 24 h exposure to the different antimicrobials in PBS. MBC values were determined in three-fold with separately grown bacterial cultures, yielding no differences in MBC values.



507

508 **FIG 2** Microscopic images of *P. aeruginosa* ATCC 39324 biofilms grown in ASM^{+}, ASM^{-} and

509 LB.

510 (a) 2D, cross-sectional OCT images, with scale bars representing 200 µm.

511 (b) CLSM 2D over-layer (left) and 3D (right) images of SYTO9 stained biofilms yielding green-

512 fluorescent bacteria. Scale bars represent 100 µm.

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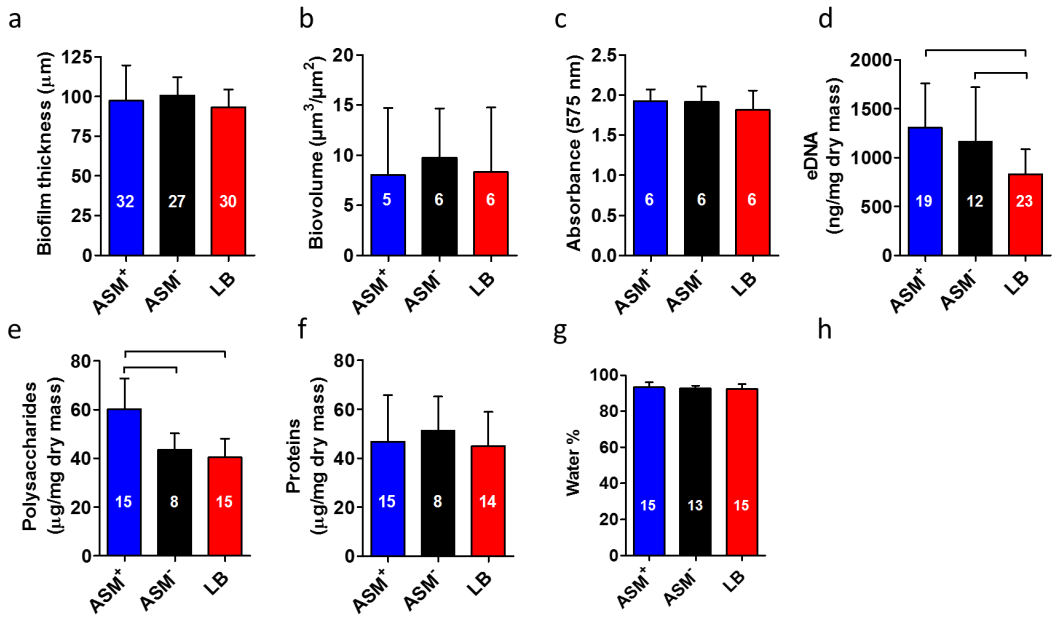


514

515 **FIG 3** Characteristics and matrix composition of *P. aeruginosa* ATCC 39324 biofilms grown in

516 ASM^+, ASM^- and LB.

517 (a) Thickness of the biofilms measured by OCT.

518 (b) Biovolume of the biofilms obtained from COMSTAT analysis of CLSM images.

519 (c) Metabolic activity of the biofilms measured with MTT.

520 (d) eDNA presence in the biofilms, isolated with phenol chloroform and measured with the

521 nanodrop-method.

522 (e) Polysaccharide presence in the biofilms measured using anthrone sulfuric acid in a

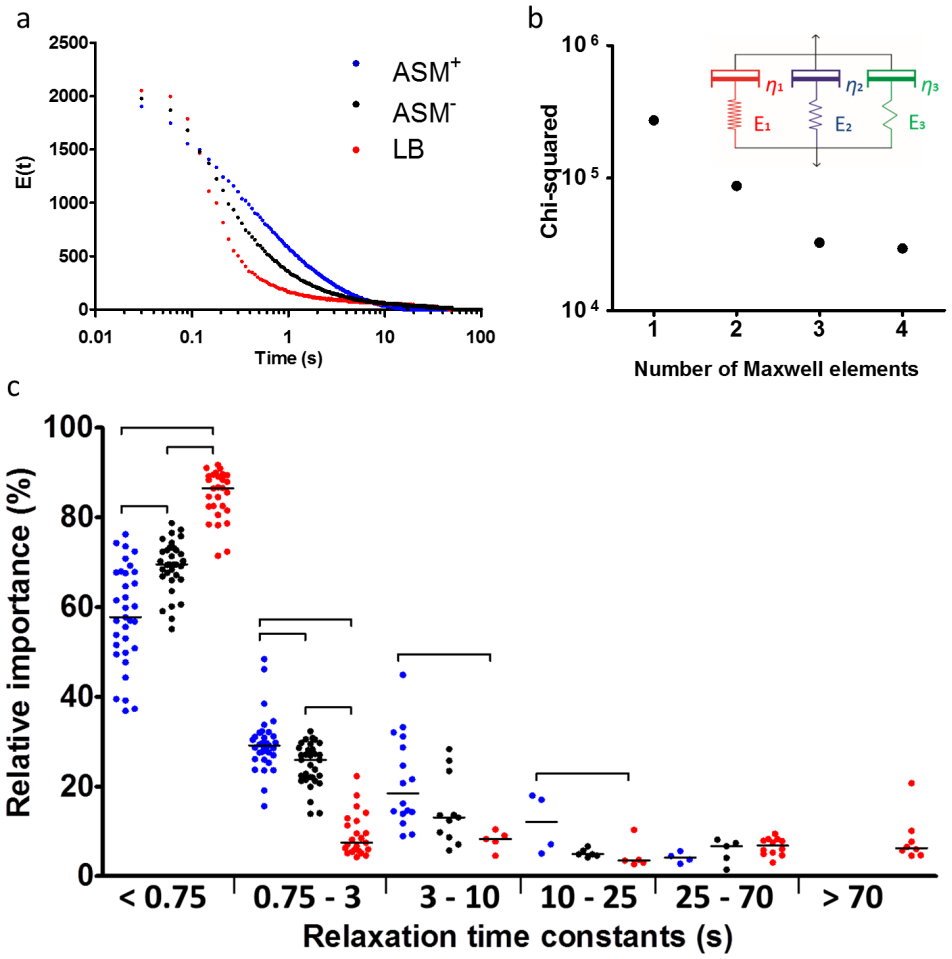
523 colorimetric assay.

524 (f) Proteins presence in the biofilm, measured with Pierce BCA Protein Assay Kit.

525 (g) Water content, obtained from a comparison of the weight of hydrated and dried biofilms and

526 expressed as a percentage of the hydrated biofilm weight.

Error bars denote standard deviations over n (numbers given in the columns) different biofilms, taken from different pans in three separate CDFF runs, except for panels b and c which were taken from different pans in two separate CDFF runs. Markers indicate significant differences ($p < 0.05$, ANOVA with Tukey post-hoc analysis) between groups.



532

533 **FIG 4** Stress relaxation analysis of *P. aeruginosa* ATCC 39324 biofilms grown in ASM$^+$, ASM$^-$

534 or LB medium.

535 (a) Examples of the normalized stress in compressed biofilms (strain 0.2) as a function of

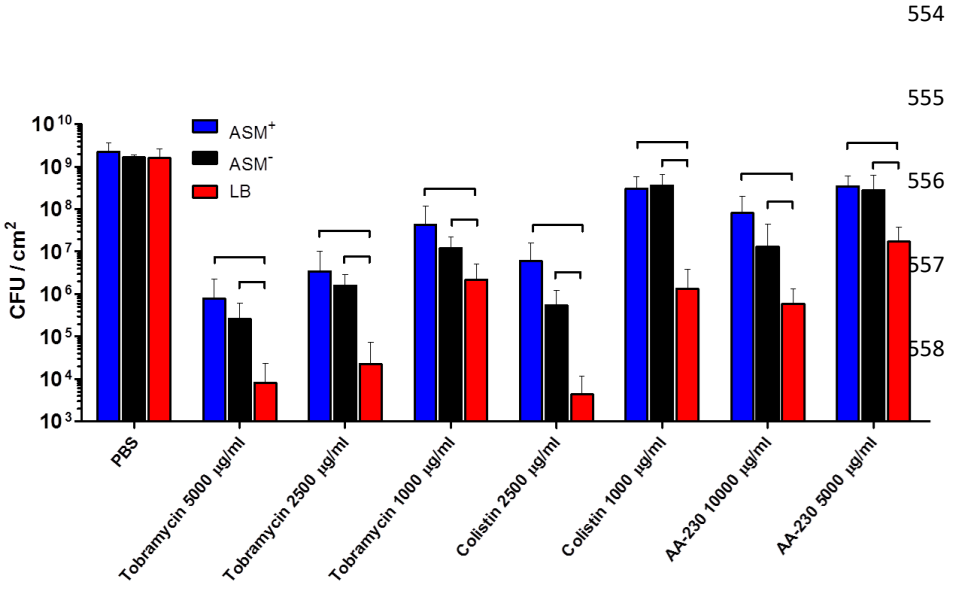
536 relaxation time. Stress at t = 0 amounted 2.2 kPa for all biofilms, regardless of growth medium.

537 (b) Quality of fitting the stress relaxation data to a generalized Maxwell model as a function of

538 the number of elements included in the model. Quality of the fit is indicated by chi-squared

539 values.

(c) Distribution of the relative importance of individual Maxwell elements (three elements model) in differently grown *P. aeruginosa* biofilms over different relaxation time constant ranges. Each data point represents a single measurement out of 30 biofilms, taking 10 biofilms from different pans in three separate CDFF runs. Median values are indicated by horizontal lines. Markers indicate significant differences ($p < 0.05$, ANOVA with Dunn's post-hoc analysis) between groups.



559 **FIG 5** The number of colony forming units per cm^2 (CFU/cm^2) in *P. aeruginosa* ATCC 39324
560 biofilms grown in ASM^+, ASM^- or LB after exposure for 24 h to different concentrations of
561 tobramycin, colistin or the antimicrobial peptide AA-230 in PBS, and including PBS as a control.
562 Error bars denote standard deviations over at least 9 different biofilms, taken from different pans
563 in three separate CDFF runs. Markers indicate significant differences ($p < 0.05$, ANOVA with
564 Tukey post-hoc analysis) between groups.

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